contamination. If fecal material is present, it should be removed before the semen is collected. Likewise, care should be taken not to introduce fecal material into the oviduct of the hen.

Subpart D—Molecular Examination Procedures

SOURCE: 72 FR 1425, Jan. 12, 2007, unless otherwise noted.

§ 147.30 Laboratory procedure recommended for the polymerase chain reaction (PCR) test for Mycoplasma gallisepticum and M. synoviae.

(a) *DNA isolation.* Isolate DNA from 1 mL of eluate from tracheal swabs in PBS or 1 mL of broth culture by a nonphenolic procedure. Centrifuge samples at 14,000 x g for 5 to 10 minutes. Decant supernatant and wash the pellet with 1 mL of PBS. Centrifuge as above and resuspend the pellet in 25–50 µl of 0.1 percent DEP (Diethyl Pyrocarbonate; Sigma) water. Boil at 120 °C for 10 minutes followed by 10 minutes incubation at 4 °C. Centrifuge as above and transfer the supernatant DNA to a nuclease-free tube. Estimate the DNA concentration and purity by spectrophotometric reading at 260 nm and 280 nm.

(b) *Primer selection.* (1) *M. gallisepticum.* The primer for *M. gallisepticum* should consist of the following sequences:

MG-F	5' GAG CTA ATC TGT AAA GTT GGT C
MG-R	5' GCT TCC TTG CGG TTA GCA AC

(2) *M. synoviae.* The primer for *M. synoviae* should consist of the following sequences:

MS–F	5' GAG AAG CAA AAT AGT GAT ATC A
MS–R	5' CAG TCG TCT CCG AAG TTA ACA A

(c) *Polymerase chain reaction.* (1) Treat each sample (100 to 2000 ng/5 µl) with one of the following 45 µl PCR cocktails:

(i) 5 µl 10x PCR buffer, 1 µl dNTP (10 mM), 1 µl of Reverse primer (50 µM), 1 µl of Forward primer (50 µM), 4 µl $MgCl_2$ (25 mM), 1 µl taq-polymerase (5 U), 32 µl DEP water.

(ii) 18 µl water, 25 µl PCR mix (Promega), 1 µl Reverse primer (50 µM), 1 µl Forward primer (50 µM).

(2) Perform DNA amplification in a Perkin-Elmer 9600 thermocycler or in a Hybaid PCR Express thermocycler.[21] The optimized PCR program is as follows:

Temperature (°C)	Duration	Cycles
94	30 seconds	30–40.
55	30 seconds	30–40.

[21] Trade names are used in these procedures solely for the purpose of providing specific information. Mention of a trade name does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or an endorsement over other products not mentioned.

Temperature (°C)	Duration	Cycles
72	1 minute	30–40.
72	5 minutes	1 (final extension).

(d) *Electrophoresis.* Mix PCR products (5 to 10 µl) with 2 µl loading buffer (Sigma) and electrophorese on a 2 percent agarose gel containing 0.5 µg/mL ethidium bromide in TAE buffer (40 mM tris; 2 mM EDTA; pH 8.0 with glacial acetic acid) for 30 minutes at 80 V. *M. gallisepticum* (185 bp) and *M. synoviae* (214 bp) amplicons can be visualized under an ultraviolet transilluminator along with the PCR marker (50 to 2000 bp; Sigma).

[72 FR 1425, Jan. 12, 2007, as amended at 74 FR 14718, Apr. 1, 2009; 76 FR 15797, Mar. 22, 2011]

§ 147.31 Laboratory procedures recommended for the real-time polymerase chain reaction test for Mycoplasma gallisepticum (MGLP ReTi).

(a) *DNA extraction.* Use Qiagen Qiamp Mini Kit for DNA extraction or equivalent validated technique/procedure. This kit utilizes the following methods: 100 µl of swab suspension incubates with 10 µl of proteinase K and 400 µl of lysis buffer at 56 °C for 10 minutes. Following incubation, 100 µl of 100 percent ethanol is added to lysate. Wash and centrifuge following extraction kit recommendations.

(b) *Primer selection.* A forward primer mglpU26 (5′-CTA GAG GGT TGG ACA GTT ATG–3′) located at nucleotide positions 765,566 to 765,586 of the *M. gallisepticum* R strain genome sequence; a reverse primer mglp164 (5′-GCT GCA CTA AAT GAT ACG TCA AA–3′) located at nucleotide positions 765,448 to 765,470 of the *M. gallisepticum* R strain genome sequence; and a Taqman dual-labeled probe mglpprobe (5′-FAM–CAG TCA TTA ACA ACT TAC CAC CAG AAT CTG–BHQ1–3′) located at nucleotide positions 765,491 to 765,520 of the *M. gallisepticum* R strain genome should be used to amplify a 139-bp fragment of the lp gene.

(c) *MGLP ReTi.* Primers and probe should be utilized in a 25 µl reaction containing 12.5 µl of Quantitect Probe PCR 2X mix (Qiagen, Valencia, CA),[22] primers to a final concentration of 0.5 µmolar, and probe to a final concentration of 0.1 µmolar, 1µl of HK–UNG Thermolabile Uracil N-glycosylase (Epicentre, Madison, WI), 2 µl of water, and 5 µl of template. The reaction can be performed in a SmartCycler (Cepheid, Sunnyvale, CA) or other equivalent validated platform procedure for real-time thermocycler at 50 °C for 2 minutes; 95 °C for 15 minutes with optics OFF; and 40 cycles of 94 °C for 15 seconds followed by 60 °C for 60 seconds with optics ON.

(d) *Determination of positive.* For each MGLP ReTi assay reaction, the threshold cycle number (CT value) was determined to be the PCR cycle number at which the fluorescence of the reaction exceeded 30 units of fluorescence. For all samples tested, any MGLP reaction that has a recorded CT value was considered positive, while any MGLP reaction that had no recorded CT value was considered negative.

(e) *Controls.* Proper controls should be used when conducting the MGLP ReTi assay as an official test of the Plan. Positive, quantitative, extraction, and internal controls are commercially available from GTCAllison, LLC, Mocksville, NC.

[74 FR 14718, Apr. 1, 2009, as amended at 76 FR 15797, Mar. 22, 2011]

Subpart E—Procedure for Changing National Poultry Improvement Plan

§ 147.41 Definitions.

Except where the context otherwise requires, for the purposes of this subpart the following terms shall be construed, respectively, to mean:

[22] Trade names are used in these procedures solely for the purpose of providing specific information. Mention of a trade name does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or an endorsement over other products not mentioned.